**Sponsor**

**Chemtura Corporation**

# Report for Acute Toxicity of MLA-3202 to Fish (*Gobiocypris rarus*)

**(Test Substance: MLA-3202)**

Study No.: S2016NC020-01

Report No.: R2016NC020-01

**Study Director: Wu Shengmin, Assistant Professor**

**Date of Report Completion**

**Feb. 17, 2017**

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# Content

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# Statement of GLP Compliance

Study No.: S2016NC020-01

Report No.: R2016NC020-01

According to "The guidelines for the testing of chemical (HJ/T 153-2004)" issued by State Environmental Protection Administration of the People's Republic of China and "OECD Guidelines for testing of chemicals", this experiment was conducted under "The guidelines of chemical testing good laboratory practices (HJ/T 175)", CMA (China Metrology Accreditation) and CNAS (China National Accreditation Service for Conformity Assessment) experimental conditions at our laboratory, and was performed in compliant with OECD Principles of Good Laboratory Practice (ENV/MC/CHEM(98)17). The experimental protocol was strictly carried out in the process of the experiment, and the present report has reflected the experimental results truly and correctly. This report only reflects the substance provided by sponsor.

Wu Shengmin — Feb. 17, 2017

(Study Director) Date:

Shi Lili — Feb. 17. 2017

(Facility management) Date:

# Quality Assurance Statement

Study No.: S2016NC020-01

Report No.: R2016NC020-01

This experiment was carried out in accordance with the experimental protocol. It is hereby certified that what the present report describes has accurately reflected the raw data of the experiment.

During the on-site process, QAU Inspections were performed for this study. The dates of Quality Assurance inspection are given below.

| Type of inspections | Phase/Process | Date | | |
|---|---|---|---|---|
| | | Start inspection | End inspection | Reporting |
| Study | Protocol | May 13,2016 | May 13,2016 | May 13,2016 |
| | Draft Report | Aug.29,2016 | Aug.29,2016 | Aug.29,2016 |
| | Final Report | Feb. 17, 2017 | Feb. 17, 2017 | Feb. 17, 2017 |
| Process | Test solutions preparation | Jun.22,2016 | Jun.22,2016 | Jun.22,2016 |
| | Observation/Determination | Jun.24,2016 | Jun.24,2016 | Jun.24,2016 |

Guo Min

(Person responsible for QAU)

Feb 17, 2017

Date:

# Study Details Page

| | |
|---|---|
| Study number: | 2016NC020-01 |
| Report number: | 2016NC020-01 |
| Study title: | Acute Toxicity of MLA-3202 to fish (*Gobiocypris rarus*) |
| Test substance[1]: | MLA-3202 |
| Chemical name: | Amides, tallow, N,N-bis(2-hydroxypropyl) |
| CAS: | 1454803-04-3 |
| Molecular formula: | $CH_{39}NO_3$ to $C_{26}H_{51}NO_3$ (in $H_2$, $C_2H_2$ and C increments) |
| Molecular weight: | NA[2] |
| Purity/Assay: | UVCB |
| Batch No.: | RC-1045 |
| Water solubility: | Trace（< 1 g/L） |
| Exp. Date: | 2017/4/11 |
| Appearance: | Liquid |
| Storage conditions: | Room Temperature |
| Facility Management: | Shi Lili, professor |
| Study Director: | Wu Shengmin, assistant professor<br>Telephone: +86 25 8528 7201<br>Facsimile: +86 25 8547 6477<br>Email: wsm@nies.org |
| Location of study: | Key Lab of Pesticide Environmental Assessment and Pollution Control, MEP (PEAPC)<br>Nanjing Institute of Environmental Sciences, MEP<br>8 Jiwang-wang-miao Street, Nanjing 210042<br>P.R. China |
| Study dates | |
| Study initial date: | Jun. 01, 2016 |
| Experiment starting date: | Jun. 12, 2016 |
| Experiment completion date: | Jun. 26, 2016 |
| Study completion date: | Feb. 17, 2017 |

[1]Test substance information was provided by the sponsor. [2]NA: The unavailable information was marked as NA.

# 1 Summary

Under static conditions, the acute toxicity of test substance (MLA-3202) to Rare minnow (*Gobiocypris rarus*) was conducted according to: "The guidelines for the testing of chemicals" (HJ/T 153-2004), "The Guidelines for the Testing of Chemicals, Effects on Biotic Systems" (the 2nd edition) (Beijing: China Environment Press. 2013), and with reference to Procedure 203 of the "Guidelines for Testing of Chemicals" of the OECD: "Fish, Acute Toxicity Test" (1992).

A range-finding test and then a definitive test were performed respectively. Nominal concentrations of 0.10, 1.00, 10.0 and 100 mg/L were used in the range-finding test. Nominal concentrations of 1%, 2%, 4%, 6%, 8% and 10% stock solution were used in the definitive test. Water samples taken from the blank control and the treatments in the definitive test were analysed. Concentrations of the test substance were quantified by GC-FID using two of the principal peaks which were considered representative of the different physico-chemical characteristics of the individual components making up the complex nature. Concentrations of the test substance were quantified using GC-FID of the measured concentrations were 0.121, 0.240, 0.503, 0.691, 0.858 and 1.27 mg/L, respectively. The analytical results showed that the concentration of the test substance was consistent in the test medium throughout the 96-hour test period (deviation within 20%). Thus a static procedure was reasonable.

In the range-finding test, 5 fish per treatment with no replicates were used. And 10 fish per treatment with no replicates were used in the definitive test. The fish were exposed for 96 hours to the test solution.

During the test period, the pH values of the control mediums and test mediums were between 7.72 and 7.97, the Dissolved Oxygen (DO) values varied from 75% ~ 97% of the air saturation at the test temperature, the temperature of the test mediums were maintained in the range of 22.8℃ to 23.1℃, and the total hardness was in the range of 164 mg (CaCO3)/L to 175 mg (CaCO3)/L. All fishes in the control group were normal. With the same conditions, $K_2Cr_2O_7$ was used as the positive control substance, and the resulting 24 h-$LC_{50}$ was 309 mg/L. So the study met the acceptability criteria prescribed by the protocol (The mortality of control ≤ 10%; pH: 6.0 ~ 8.5; dissolved oxygen concentration: >60% of the air saturation value; total hardness: 10 ~ 250 mg ($CaCO_3$)/L; temperature: (23±2)℃; 24 h-$LC_{50}$ of $K_2Cr_2O_7$ in the range of 200 mg/L to 400 mg/L). Therefore the test was considered valid.

During the test period, all fish in the control and treatment of 0.121, 0.240 mg/L were alive and appeared normal. Effects (Fish lying on side or back) occurred at concentrations of 0.503 mg/ and above. All fish were dead after 96h-exposure at concentration of 0.858, 1.27 mg/L. The results showed that under valid static test conditions, the 96 h-$LC_{50}$ of the test substance to fish (Rare minnow, *Gobiocypris rarus*) was 0.500 mg/L, with 95% confidence limit of 0.420 mg/L ~ 0.600 mg/L (based on the measured concentration). The maximum tested concentration causing no mortality (96 h-$LC_0$) was 0.240 mg/L. The minimum concentration causing 100% mortality (96 h-$LC_{100}$) is 0.858 mg/L, i.e.:

96 h-$LC_{50}$ = 0.500 mg/L, 95% CI 0.420 ~ 0.600 mg/L (based on measured concentration);

96 h-$LC_0$ = 0.240 mg/L (measured concentration);

96 h-$LC_{100}$ = 0.858 mg/L (measured concentration).

# 2 Test Purpose

This test was designed to determine the acute toxicity of test substance (MLA-3202) to fish (*Gobiocypris rarus*). The test fishes were exposed for 96 hours to the test solution. The study comprised at least one range-finding test followed by a definitive test to determine the 96-hour median lethal concentration ($LC_{50}$) and 95% confidence limit of the test substance.

The study met the requirements of:

[1] HJ/T 153-2004, The guidelines for the testing of chemicals [S]. Beijing: SEPA, 2004

[2] CRC-MEP. The Guidelines for the Testing of Chemicals, Effects on Biotic Systems [M]. 2nd edition. Beijing: China Environment Press. 2013: 30-36.

[3] GB/T 27861-2011, Chemicals-Fish acute toxicity test, Beijing: SAC, 2011.

[4] GB/T 29763-2013，Chemicals-Rare Minnow (*Gobiocypris rarus*) acute toxicity test Beijing: SAC, 2013.

[5] Guideline for the testing of chemicals 203. Fish, Acute Toxicity Test[S]. OECD, 1992.

[6] Ecological Effects Test Guidelines, OPPTS 850.1075. Fish Acute Toxicity Test, Freshwater and Marine[S]. EPA 712-C-96-118, 1996.

[7] EC Directive 2001/59,Part C.1 Acute Toxicity for Fish. O. J. L142, 2008

**The Guide for Care and Use of Laboratory Animals (1988)**

The in-life experimental procedures undertaken during the course of this study were subject to the provisions of the Guide for Care and Use of Laboratory Animals (1988) in China. The Guide, administered by the Ministry of Science and Technology of the People's Republic of China, regulates all scientific procedures in living animals which may cause pain, suffering, distress or lasting harm and provides for the designation of establishments where procedures may be undertaken, the licensing of trained individuals who perform the practical techniques and the issue of project licences for specified programmes of work.

This study complied with all applicable sections of the Guide and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures.

The number of animals used was the minimum that was consistent with scientific integrity and regulatory acceptability, consideration having been given to the welfare of individual animals in terms of the number and extent of procedures to be carried out on each animal.

# 3 Equipments & Materials

## 3.1 Test Organism

The test species Rare minnow, *Gobiocypris rarus* (Batch No. F20160415G), were obtained from a fish supplier of Institute of Hydrobiology, Chinese Academy of Sciences.

Fish were held at least for 12 days in holding tanks supplied with a continuous flow of aerated water before being used for testing. Fish to be used in the test were held for 7 days in water of the quality and temperature to be used in the test.

A photoperiod of 16 hours light, provided by overhead fluorescent tubes, and 8 hours dark was maintained. The oxygen concentration was more than 60% of the air saturation value.

The fish was fed daily during the holding period on proprietary fish food. They were held without food for approximately 24 hours before being placed into the test vessels. The ingredients of the fish food are given as follows:

Crude Protein > 36.0%

Crude Fat > 2.0%

Crude Fibre < 3.0%

Crude Ash < 13.5%

Moisture < 10.0%

Characteristics of the fish food are measured at least twice a year by Jiangsu Provincial Center for Disease Prevention and Control. The latest measuring result is showed in Table 11.

During the holding period the tanks was inspected daily and any debris or unhealthy or dead fish removed.

After the 48 hour settling-in period, no mortality was observed in the following 7 days. So the batch of fish (Batch No.: F20160415G) was accepted.

The average wet-weight and length of the fish used to the test was 0.222 g and 2.75 cm, and the relative standard deviation (RSD) was 7.75% and 4.61%, respectively (See Table 1).

**3.2 Dilution Medium**

Good quality tap water which had been dechlorinated for at least 24 hours was used. The total hardness of the dilution water was 185 mg ($CaCO_3$)/L and pH was 8.17 at room temperature. Characteristics of the dilution water are measured at least twice a year by Jiangsu Provincial Center for Disease Prevention and Control. The latest measuring result is showed in Table 10.

**3.3 Apparatus**

Normal laboratory apparatus:

1) Oxygen meter, Thermometer and pH meter (HACH HQ40d);
2) Equipment for determination of hardness of water (HACH 16900);
3) Analytic Balance (MS105DU, Accuracy 0.1 mg, METTLER TOLEDO, Switzerland);
4) Tanks made of glass material, with a sealable inert lid, and with a capacity of approximately 5 L (Haimen Sanhe Zuping Glass Instrument Factory, Jiangsu);
5) Thermosemi-static water bath (Chang Yuan Medical Instrument Factory, Jiangsu).
6) GC-FID: Agilent 7820A (Agilent, USA)

**3.4 Reference Substance**

Reference substance: Potassium dichromate, $K_2Cr_2O_7$, CAS: 7778-50-9; purity: ≥99.8%; Lot number: LVC0052; Beijing Leon Technology Co. Ltd.

The test with the reference substance is performed at least once each batch of fish as a means of assuring that the laboratory test conditions are adequate and have not changed significantly (Study No.:S2016RI001-03). The recently results of this study

are shown in Table 6.

# 4 Test Method

## 4.1 Preparation of the Test Solutions

In the range-finding test, the test solution was prepared by directly adding appropriate amounts of MLA-3202 in dilution water and then facilitating its dispersion by stirring for 30 min. The 0.10 mg/L and 1.00 mg/L test solutions were colourless and clear. The 10.0, 100 mg/L test solutions were insufficiently soluble and turbid.

In the definitive test, the test medium was prepared as a slow stir stock solution. The test solution was prepared by adding 1.0035 g MLA-3202 in 10 L dilution water. The aqueous test substance mixture was stirred for 1 hrs on a magnetic stirplate and a telfon stirbar at 35℃. The vortex height was set at least 10% of the liquid height. At the end of the 1 h, stirring was stopped. The stock solution stood for 2 hour at room temperature prior to the removal of any undissolved test item by filtration through 0.45 μm millipore membrane to produce the stock solution of the test item. The test solutions were diluted by the stock solution.

The details of the test solutions preparation were as follows:

| Test Type | Nominal Concentration (mg/L) | Amount of Test Substance Added | Dilution Medium Volume (L) |
|---|---|---|---|
| Range-finding Test | 0 (Blank control) | 0 | 3 |
| | 0.10 | 0.0003 g | 3 |
| | 1.00 | 0.0035 g | 3 |
| | 10.0 | 0.0309 g | 3 |
| | 100 | 0.3023 g | 3 |
| Definitive Test | Stock solution | 1.0035 g | 10 (filtrated) |
| | 0 (Blank control) | 0 | 3 |
| | 1% stock solution | 30 mL stock solution | 3 |
| | 2% stock solution | 60 mL stock solution | 3 |
| | 4% stock solution | 120 mL stock solution | 3 |
| | 6% stock solution | 180 mL stock solution | 3 |
| | 8% stock solution | 240 mL stock solution | 3 |
| | 10% stock solution | 300 mL stock solution | 3 |

## 4.2 Observations and Evaluations

During the test, all kinds of abnormal responses of the fish observed were recorded, such as mortality, inactivity, abnormal swimming pattern, other abnormal behaviour, etc. Fish were considered dead if there was no visible movement (e.g. gill movements) and if touching of the caudal peduncle produced no reaction.

## 4.3 Range-finding Test

The range-finding test, carried out under static conditions, was conducted to determine the range of concentrations for the subsequent test.

In the range-finding test, groups of fish (5 per group) were exposed to the test solutions

with nominal concentration of 0.10, 1.00, 10.0 and 100 mg/L. One control group was also included in the study using test water without the test substance. For each test tank 3 L test solution was filled in. No replicates were used.

The test fish were randomly chosen and put in appropriate test solutions after the temperature had been adjusted to the required value. This was done in 30 minutes.

During the test, the following conditions were maintained:

-Light: 16 hours photoperiod daily (light intensity: 1000 to 1500 lux);

-Temperature: 23.0℃ to 23.5℃;

-Oxygen concentration: 80% ~ 95% of the air saturation; No aeration.

-Feeding: none.

The test duration was 96 hours. Dead fish was removed at least once daily and discarded. The mortalities of the fish were recorded at 24, 48, 72 and 96 h, and then the maximum concentration causing no mortality (96 h-$LC_0$) and the minimum concentration causing 100 % mortality ($LC_{100}$) were determined.

**4.4 Definitive Test**

A static method was adopted in definitive test. The stability of the test solution is confirmed by results indicated in Table 4 (deviation within 20%). Based on the results of the range-finding test, the concentration of 1%, 2%, 4%, 6%, 8% and 10% stock solution was assigned in definitive test. Synchronously a blank control was used in the test. No replicate was assigned for each treatment group and control group, while the initial number of testing fish was 10 for each group.

The test fish were randomly chosen and put in different test solutions after the temperature has been adjusted to the required value. This was done in 30 minutes.

During the test, the following conditions were maintained:

-Light: 16 hours photoperiod daily (light intensity: 1000 lux to 1500 lux);

-Temperature: 22.8° C to 23.1° C;

-Oxygen concentration: 75% ~ 97% of the air saturation; No aeration.

-Feeding: none.

At 3, 6, 24, 48, 72 and 96 h, the mortalities of the fish were recorded, and observations on individual behaviour were performed. Meanwhile, measurements of pH, dissolved oxygen and temperature were carried out and recorded daily.

**4.5 Validity of Test**

(1) Control group

A control group, comprising the same number of fish as that exposed at each test concentration, was placed into test water alone.

(2) Reference substance test

With the conditions maintained as before, $K_2Cr_2O_7$ was used as the test substance and the resulting 24 h-$LC_{50}$ was 309 mg/L. The results of this study are shown in Table 6.

(3) Fish loading

0.7 g to 0.9 g fish (wet weight) per litre of test medium.

(4) Validity of test result

During the test period, the pH values of the control mediums and test mediums were between 7.72 and 7.97, and the Dissolved Oxygen (DO) values varied from 75% ~ 97% of the air saturation at the test temperature, the temperature of the test mediums were maintained in the range of 22.8°C to 23.1°C, and the total hardness was in the range of 164 to 175 mg ($CaCO_3$)/L. During the test, all fishes in the control group were normal. With the same conditions, $K_2Cr_2O_7$ was used as the positive control substance, and the resulting 24 h-$LC_{50}$ was 309 mg/L. So the study met the acceptability criteria prescribed by the protocol (The mortality of control ≤ 10%; pH: 6.0 ~ 8.5; dissolved oxygen concentration: > 60% of the air saturation value; total hardness: 10 ~ 250 mg ($CaCO_3$)/L; temperature: (23 ±2)°C; 24 h-$LC_{50}$ of $K_2Cr_2O_7$ in the range of 200 to 400 mg/L). Therefore the test was considered valid.

**4.6 Stability of Test Solution and Chemical Analysis**

(1) Preparation of standard stock solution

A standard stock solution I of the test substance (1152 mg/L) was prepared by dissolving 0.0576 g test substance into 50.0 mL n-hexane.

The standard solution II of 115 mg/L were prepared by drawing 1.00 mL above standard stock solution (1152 mg/L) to 10.0 mL with n-hexane.

(2) Working solution

The working solutions were prepared by drawing appropriate amounts standard solution to 10.0 mL with n-hexane. Details of the working solutions are showed as follows:

| Concentration (mg/L) | Concentration of Standard Solution Added (mg/L) | Volume of Standard Solution Added (mL) | Final Volume after Dilution (mL) |
|---|---|---|---|
| 0.00 | 115 | 0.00 | 10.0 |
| 5.76 | 115 | 0.50 | 10.0 |
| 11.5 | 115 | 1.00 | 10.0 |
| 23.0 | 115 | 2.00 | 10.0 |
| 46.1 | 115 | 4.00 | 10.0 |
| 92.2 | 115 | 8.00 | 10.0 |
| 115 | 115 | 10.0 | 10.0 |

(3) GC-FID conditions

Apparatus: GC (Agilent 7820A, USA) with FID detector

Column: Agilent HP-5 30 m×0.32 mm ID 0.25 μm

Injection temperature: 300°C

Mode: No split

Oven temperature: 200°C → 280°C (5°C/min) →300°C (3min, 20°C/min)

Detector: FID 300°C

$H_2$ flow: 40 mL/min

Air flow: 400 mL/min

Make up gas ($N_2$): 16 mL/min

Column flow rate: 1.2 mL/min

Injection volume: 1 μL

Under the above conditions, the retention time was about 3.1 min, 3.4min, 3.6min, 4.5min and 5.1 min (see Fig. 2)

(4) Sampling and analysis of the test solution

100 mL water samples were taken (at least in duplicate) from each concentration during the definitive test at 0, 24, 48, 72 and 96 h. On each occasion, one sample was analysed after certain pre-treatments; the remaining samples were retained in case further analysis would be required.

(5) Pre-treatment method of the sample

100 mL collected water samples were extracted with 50mL dichloromethane for 15 min and repeated once, combined twice organic phase. The organic phase was concentrated by rotary evaporation to near dryness. The concentrate was diluted to total volume to 1.00 mL with n-hexane (concentrated 100 times), then the dilution was analysed by GC-FID

# 5 Data Processing

Trimmed Spearman-Karber Method (Version 1.5, USEPA) were used to calculate the $LC_{50}$ and 95% confidence limits.

# 6 Results

## 6.1 Analytical Method for Determination of the test substance in Water

(1) Specificity

Under the GC-FID condition, at the retention time of 3.1 min, 3.4min, 3.6min, 4.5min and 5.1 min, the chromatographic peak emerged for the test substance sample (Figure 2) and there was no chromatographic peak emerged for the blank sample (Figure 4). So the GC-FID method was specific for MLA-3202.

(2) Calibration curve

A series of standard solutions with concentration at 0.00, 5.76, 11.5, 23.0, 46.1, 92.2 and 115 mg/L were measured under the GC-FID conditions mentioned above. Concentrations of the test substance were quantified by GC-FID using two of the principal peaks which were considered representative of the different physico-chemical characteristics of the individual components making up the complex nature. Based on the test result, a linear regression equation was obtained between the concentration and the GC-FID response: $A$=3864.7$c$+454.47, with good linearity of $r^2$ = 0.9975, where $A$ represents peak area (μV*s); and $c$ is the concentration of the test substance (mg/L) (See Figure 1). The results show that linearity for concentration range of 0.00 mg/L to 115 mg/L is good.

(3) Precision

Under the above condition, 115 mg/L standard solutions of the test substance were

analysed for 6 times, the results were shown in Table 2. The relative standard deviations were 2.97%.

(4) Recovery Test

The recovery samples with concentrations of 0.115 mg/L were prepared by adding 0.10 mL standard solution II (115 mg/L) to a total volume of 100 mL test water with three replications. 100 mL recovery samples were extracted with 50 mL dichloromethane for 15 min and repeated once, combined twice organic phase. The organic phase was concentrated by rotary evaporation to near dryness. The concentrate was diluted to total volume to 1.00 mL with n-hexane (concentrated 100 times), then the dilution was analysed by GC-FID. Measurements obtained from the recovery test were shown in Table 3. The recovery rate was 80.9% ~ 88.7% for the concentration of 0.115 mg/L. The relative standard deviation was 4.66%.

The recovery samples with concentrations of 1.15 mg/L were prepared by adding 0.10 mL standard solution I (1152 mg/L) to a total volume of 100 mL test water with three replications. 100 mL recovery samples were extracted with 50 mL dichloromethane for 15 min and repeated once, combined twice organic phase. The organic phase was concentrated by rotary evaporation to near dryness. The concentrate was diluted to total volume to 1.00 mL with n-hexane (concentrated 100 times), then the dilution was analysed by GC-FID. Measurements obtained from the recovery test were shown in Table 3. The recovery rate was 88.7% ~ 94.8% for the concentration of 1.15 mg/L. The relative standard deviation was 3.34%.

(5) Limit of Detection (LOD) and Limit of Quantification (LOQ)

If the calculation is based on S/N≥3, the LOD is 2.00 mg/L. If the calculation is based on S/N≥10, the LOQ is 5.00 mg/L.

In this analytical method, the minimum detection concentration for water sample is 0.05 mg/L.

**6.2 Analysis of the test substance in Test Solutions**

The analytical results for the test samples from the definitive test are given in Table 4. Figure 4 and Figure 5 are the GC-FID chromatogram of the control sample and test solution. The measured concentrations of 1%, 2%, 4%, 6%, 8% and 10% stock solution were 0.121, 0.240, 0.503, 0.691, 0.858 and 1.27 mg/L, respectively. The results indicated that concentration of test substance was stable (deviation within 20%) in the water during the test period. Thus static method used in the definitive test was reasonable.

**6.3 Test Condition**

During the definitive test, the pH, dissolved oxygen concentration, total hardness and temperature of the control and treatment groups were showed in Table 5.

During the whole test period, the pH values of the control mediums and test mediums were between 7.72 and 7.97, the Dissolved Oxygen (DO) values varied from 75% ~ 97% of the air saturation, the temperature of the test mediums was maintained in the range of 22.8℃ to 23.1℃, and the total hardness was in the range of 164 to 175 mg ($CaCO_3$)/L.

**6.4 Mortality and Effects**

Table 7 and Table 8 show the mortality data during the range-finding test and definitive test respectively. Table 9 shows the summary of the visual observations (for behaviour or abnormalities) during the definitive test. During the test period, all fish in the control and treatment of 1% stock solution (measured concentration 0.121 mg/L) and 2% stock solution (measured concentration 0.240 mg/L) were alive and appeared normal. Effects (Fish lying on side or back) occurred at concentrations of 4% stock solution (measured concentration 0.503 mg/L) and above. All Fish were dead after 96h-exposure at concentration of 8% stock solution (measured concentration 0.858 mg/L) and 10% stock solution (measured concentration 1.27 mg/L).

### 6.5 Conclusions

The results showed that under valid static test conditions, the 96 h-$LC_{50}$ of the test substance to fish (Rare minnow, *Gobiocypris rarus*) was 0.500 mg/L, with 95% confidence limit of 0.420 mg/L ~ 0.600 mg/L (based on the measured concentration). The maximum tested concentration causing no mortality (96 h-$LC_0$) was 0.240 mg/L. The minimum concentration causing 100% mortality (96 h-$LC_{100}$) was 0.858 mg/L, i.e.:

96 h-$LC_{50}$ = 0.500 mg/L, 95%CI 0.420 ~ 0.600 mg/L (based on measured concentration);

96 h-$LC_0$ = 0.240 mg/L (measured concentration);

96 h-$LC_{100}$ = 0.858 mg/L (measured concentration).

## 7 Deviations

None

## 8 Health & Safety

In order for PEAPC to comply with Law of the People's Republic of China on the Prevention and Treatment of Occupational Diseases 2001, and the current Control of Substances Hazardous to Health Regulations, it is a condition of undertaking the study that the Sponsor provide PEAPC with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to PEAPC. The Sponsor also complied with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed PEAPC test substance data sheet must be received at PEAPC before the test substance can be handled in the laboratory.

## 9 Maintenance of Records & Documentation

All raw data arising from the performance of this study will remain the property of the Sponsor.

Records and documentation relating to this study (including the Study Protocol, raw data and a copy of the final report) will be maintained for a period of ten years from the date on which the Study Director signs the final report. Remaining samples will be retained by test facility in its archive for a period of one year from the date on which the Study Director signs the final report. After this one year, if no request of sponsor on return or further retention of the materials, the retained samples will be disposed as hazardous waste

process.

Test report in 3 copies with original signatures will be provided. 1 of them will be retained by test facility in its archive, and the remaining 2 will be sent to sponsor. The Quality Assurance records relevant to this study will also be archived.

## 10 References

[1] HJ/T 153-2004, The guidelines for the testing of chemicals [S]. Beijing: SEPA, 2004

[2] CRC-MEP. The Guidelines for the Testing of Chemicals, Effects on Biotoc Systems [M]. 2nd edition. Beijing: China Environment Press. 2013: 30-36.

[3] GB/T 27861-2011, Chemicals-Fish acute toxicity test, Beijing: SAC, 2011.

[4] GB/T 29763-2013, Chemicals-Rare Minnow (Gobiocypris rarus) acute toxicity test Beijing: SAC, 2013.

[5] Guideline for the testing of chemicals 203. Fish, Acute Toxicity Test. OECD, 1992.

[6] Ecological Effects Test Guidelines, OPPTS 850.1075. Fish Acute Toxicity Test, Freshwater and Marine. EPA 712-C-96-118, 1996.

[7] EC Directive 2001/59,Part C.1 Acute Toxicity for Fish. O. J. L142, 2008

[8] Hamilton, M A, Russo C R and Thurston V R. Trimmed Spearman-Karber Method for Estimating Median Lethal Concentrations in Toxicity Bioassays. Environmental Science & Technology. 1997, 11(7): 714-719.

## Tables

**Table 1 Individual and Mean Fish Weights and Lengths**

| No. of the Subsample Fish | Length (cm) | Weight (g) |
|---|---|---|
| 1 | 2.92 | 0.236 |
| 2 | 2.87 | 0.203 |
| 3 | 2.64 | 0.247 |
| 4 | 2.52 | 0.214 |
| 5 | 2.71 | 0.197 |
| 6 | 2.66 | 0.233 |
| 7 | 2.83 | 0.219 |
| 8 | 2.89 | 0.207 |
| 9 | 2.74 | 0.243 |
| 10 | 2.73 | 0.224 |
| Mean | 2.75 | 0.222 |
| RSD (%) | 4.61 | 7.75 |

**Table 2 Precision of the GC-FID method for the test substance**

| Nominal Concentration (mg/L) | Peak Area (uv.s) | RSD (%) |
|---|---|---|
| 115 | 392833 | 2.97 |
| | 419873 | |
| | 403026 | |
| | 420642 | |
| | 423510 | |
| | 403533 | |

**Table 3 Recovery of the test substance in Recovery Sample**

| Add. Concentration (mg/L) | Measured Concentration (mg/L) | Recovery (%) | Mean Recovery (%) | RSD (%) |
|---|---|---|---|---|
| 0.115 | 0.102 | 88.7 | 85.2 | 4.66 |
| | 0.099 | 86.1 | | |
| | 0.093 | 80.9 | | |
| 1.15 | 1.05 | 91.3 | 91.6 | 3.34 |
| | 1.09 | 94.8 | | |
| | 1.02 | 88.7 | | |

**Table 4 Stability Test Results of the test substance in Test Medium (Definitive Test)**

| Nominal Concentration | Measured Concentration (mg/L) | | | | | |
|---|---|---|---|---|---|---|
| | 0 h | 24 h | 48 h | 72 h | 96 h | Mean |
| 0 (Blank control) | ND[1] | ND | ND | ND | ND | — |
| 1% stock solution | 0.130 | 0.124 | 0.120 | 0.119 | 0.111 | 0.121 |
| 2% stock solution | 0.260 | 0.254 | 0.241 | 0.227 | 0.219 | 0.240 |
| 4% stock solution | 0.536 | 0.525 | 0.505 | 0.488 | 0.463 | 0.503 |
| 6% stock solution | 0.751 | 0.711 | 0.695 | 0.660 | 0.639 | 0.691 |
| 8% stock solution | 0.932 | 0.875 | 0.842 | 0.833 | 0.808 | 0.858 |
| 10% stock solution | 1.33 | 1.29 | 1.20 | NA | NA | 1.27 |

[1]ND: less than LOQ. [2]NA: not detected due to all test fish death on the day before

**Table 5 Water Quality Parameters of Test Solutions during the Definitive Test**

| Nominal Concentration | Duration (h) | pH | Temperature (°C) | Dissolved oxygen (%) | Hardness (mg/L$CaCO_3$) |
|---|---|---|---|---|---|
| 0 (Blank control) | 0 | 7.72 | 23.1 | 93 | 171 |
| | 24 | 7.77 | 23.0 | 89 | 170 |
| | 48 | 7.75 | 22.8 | 85 | 167 |
| | 72 | 7.79 | 22.9 | 81 | 173 |
| | 96 | 7.85 | 23.1 | 77 | 165 |
| 1% stock solution | 0 | 7.79 | 23.1 | 95 | 173 |
| | 24 | 7.80 | 23.0 | 90 | 166 |
| | 48 | 7.78 | 22.8 | 87 | 169 |
| | 72 | 7.83 | 22.9 | 83 | 175 |
| | 96 | 7.91 | 23.1 | 79 | 165 |
| 2% stock solution | 0 | 7.81 | 23.1 | 96 | 168 |
| | 24 | 7.84 | 23.0 | 92 | 164 |
| | 48 | 7.85 | 22.8 | 87 | 169 |
| | 72 | 7.89 | 22.9 | 82 | 174 |
| | 96 | 7.90 | 23.1 | 76 | 172 |
| 4% stock solution | 0 | 7.80 | 23.1 | 94 | 166 |
| | 24 | 7.83 | 23.0 | 88 | 172 |
| | 48 | 7.87 | 22.8 | 83 | 171 |
| | 72 | 7.94 | 22.9 | 80 | 175 |
| | 96 | 7.94 | 23.1 | 75 | 168 |
| 6% stock solution | 0 | 7.84 | 23.1 | 97 | 169 |
| | 24 | 7.87 | 23.0 | 91 | 171 |
| | 48 | 7.90 | 22.8 | 85 | 168 |
| | 72 | 7.96 | 22.9 | 80 | 169 |
| | 96 | 7.97 | 23.1 | 76 | 167 |
| 8% stock solution | 0 | 7.85 | 23.1 | 95 | 169 |
| | 24 | 7.92 | 23.0 | 90 | 173 |
| | 48 | 7.90 | 22.8 | 86 | 168 |
| | 72 | 7.91 | 22.9 | 82 | 172 |
| | 96 | NA | NA | NA | NA |
| 10% stock solution | 0 | 7.87 | 23.1 | 96 | 167 |
| | 24 | 7.90 | 23.0 | 92 | 168 |
| | 48 | NA | NA | NA | NA |
| | 72 | NA | NA | NA | NA |
| | 96 | NA | NA | NA | NA |

NA: not detected due to all test fish death on the day before.

**Table 6 Toxicity of Potassium Dichromate to *Gobiocypris rarus*** (Study No.:S2016RI001-03)

| Nominal Concentration (mg/L) | Initial Number of Fish | The Number of the Dead Fish | | | |
|---|---|---|---|---|---|
| | | 6 h | 12 h | 18 h | 24 h |
| 0 | 10 | 0 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 0 | 0 |
| 100 | 10 | 0 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 0 | 0 |
| 200 | 10 | 0 | 0 | 1 | 2 |
| | 10 | 0 | 0 | 1 | 2 |
| | 10 | 0 | 0 | 1 | 2 |
| 300 | 10 | 0 | 1 | 2 | 3 |
| | 10 | 0 | 1 | 2 | 3 |
| | 10 | 0 | 1 | 2 | 3 |
| 400 | 10 | 2 | 3 | 5 | 6 |
| | 10 | 2 | 3 | 5 | 6 |
| | 10 | 2 | 3 | 5 | 6 |
| 500 | 10 | 4 | 5 | 8 | 10 |
| | 10 | 4 | 5 | 8 | 10 |
| | 10 | 4 | 5 | 8 | 10 |
| $LC_{50}$ (mg/L) | | - | 500 | 393 | 309 |
| 95% confidence limit (mg/L) | | - | - | 337~459 | 256 ~ 374 |

**Table 7 Mortality during the Range-finding Test**

| Nominal Concentration (mg/L) | Initial Number of Fish | The Number of the Dead Fish | | | |
|---|---|---|---|---|---|
| | | 24 h | 48 h | 72 h | 96 h |
| 0 (Blank control) | 5 | 0 | 0 | 0 | 0 |
| 0.10 | 5 | 0 | 0 | 0 | 0 |
| 1.00 | 5 | 3 | 4 | 5 | 5 |
| 10.0 | 5 | 5 | 5 | 5 | 5 |
| 100 | 5 | 5 | 5 | 5 | 5 |

**Table 8 Mortality during the Definitive Test**

| Nominal Concentration | Measured Concentration (mg/L)[1] | Initial Number of Fish | The Number of the Dead Fish | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 3 h | 6 h | 24 h | 48 h | 72 h | 96 h |
| 0 (Blank Control) | ND[2] | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1% stock solution | 0.121 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2% stock solution | 0.240 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4% stock solution | 0.503 | 10 | 0 | 0 | 0 | 1 | 3 | 4 |
| 6% stock solution | 0.691 | 10 | 0 | 0 | 1 | 3 | 6 | 8 |
| 8% stock solution | 0.858 | 10 | 0 | 3 | 6 | 8 | 10 | 10 |
| 10% stock solution | 1.27 | 10 | 4 | 7 | 10 | 10 | 10 | 10 |
| $LC_{50}$ (mg/L, based on the measured concentration) | | | | | 0.850 | 0.720 | 0.560 | 0.500 |
| 95% confidence limit (mg/L, based on the measured concentration) | | | | | 0.760~0.940 | 0.620~0.830 | 0.470~0.670 | 0.420~0.600 |

[1] The average of the measured concentrations at 0, 24, 48, 72, 96 h. [2] ND: not detected.

**Table 9 Visual Observations during the Definitive Test**

| Nominal Concentration | Measured Concentration (mg/L) | Visual Observations | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3 h | 6 h | 24 h | 48 h | 72 h | 96 h |
| 0 (Blank Control) | ND[2] | 10NB | 10NB | 10NB | 10NB | 10NB | 10NB |
| 1% stock solution | 0.121 | 10NB | 10NB | 10NB | 10NB | 10NB | 10NB |
| 2% stock solution | 0.240 | 10NB | 10NB | 10NB | 10NB | 10NB | 10NB |
| 4% stock solution | 0.503 | 10NB | 10NB | 8NB&2SR | 7NB&2SR& 1dead | 4NB&3SR& 3dead | 4NB&2SR& 4dead |
| 6% stock solution | 0.691 | 10NB | 9NB&1SR | 8NB&1SR& 1dead | 5NB&2 SR&3dead | 2 NB&2 SR&6 dead | 2SR&8dead |
| 8% stock solution | 0.858 | 7NB&3SR | 5NB&2SR& 3dead | 1NB&3SR& 6dead | 2SR&8dead | 10dead | 10dead |
| 10% stock solution | 1.27 | 4 NB&2 SR&4dead | 3SR&7dead | 10dead | 10dead | 10dead | 10dead |

Note: NB—Normal behaviours; SR—Fish lying on side or back.

**Table 10 Characteristics of the Dilution Water**

| Items | Analyzed value | Acceptable value* |
|---|---|---|
| As | <0.001 mg/L | ≤ 0.001 mg/L |
| Cd | 0.0001 mg/L | ≤ 0.0001 mg/L |
| Pb | <0.001 mg/L | ≤ 0.001 mg/L |
| Co | <0.001 mg/L | ≤ 0.001 mg/L |
| Ni | < 0.001 mg/L | ≤ 0.001 mg/L |
| Hg | < 0.0002 mg/L | ≤ 0.0005 mg/L |
| Cu | < 0.01mg/L | ≤ 0.01 mg/L |
| $Cr^{6+}$ | < 0.004 mg/L | ≤ 0.01 mg/L |
| Fe | <0.01 mg/L | ≤ 0.3 mg/L |
| Zn | 0.055 mg/L | ≤ 1.0 mg/L |
| Ag | < 0.001 mg/L | ≤ 0.05 mg/L |
| pH | 8.17 | 6.0 ~ 8.5 |
| The total hardness | 184.9 mg($CaCO_3$)/L | 10 ~ 250 mg ($CaCO_3$)/L |
| Visible substance | NA | ≤ 5 mg/L |
| Malathion | < 0.025 mg/L | ≤ 0.005 mg/L |
| Methyl parathion | < 0.002 mg/L | ≤ 0.002 mg/L |
| Parathion | < 0.003 mg/L | ≤ 0.003 mg/L |
| Total organic carbon (TOC) | 1.62 mg/L | ≤ 2 mg/L |

Note: Acceptable values refer to the standards as follow: TG 203: "Fish, Acute Toxicity Test" (1992), TG 305: Bioaccumulation in Fish (1996), Water quality standard for fishes (GB 11607-89), Environmental quality standards for surface water (GB3838-2002), which of II water quality standard (apply to centralized drinking water and surface water source protection areas, rare aquatic habitat, fish and shrimp spawning grounds, larvae feeding grounds, etc.).

**Table 11 Characteristics of the Fish Food**

| Items | Analyzed value | Acceptable value |
|---|---|---|
| Crude Protein | 40.7% | ≥ 36% |
| Crude Fat | 7.7% | ≥ 2% |
| Ca | 1.4% | ≤ 2.0% |
| BHC | < 0.002 mg/kg | < 0.002 mg/kg |
| DDT | < 0.004 mg/kg | < 0.004 mg/kg |
| Hg | 0.020 mg/kg | < 0.02 mg/kg |
| Al | 210.8 mg/kg | ≤ 500 mg/kg |
| Fe | 552 mg/kg | - |
| Pb | 0.39 mg/kg | ≤ 1.0 mg/kg |
| As | 0.66 mg/kg | ≤ 1.0 mg/kg |
| Cu | 35.7 mg/kg | ≤ 100 mg/kg |
| Zn | 253 mg/kg | ≤ 300 mg/kg |
| Cr | 0.82 mg/kg | ≤ 1.0 mg/kg |
| Ni | 0.66mg/kg | ≤ 1.0 mg/kg |
| Sb | 0.048mg/kg | < 0.10 mg/kg |
| K | 10848 mg/kg | - |
| Na | 9186 mg/kg | - |
| Se | <0.05 mg/kg | ≤ 1.0 mg/kg |
| Mg | 3306 mg/kg | - |
| Methyl parathion | < 0.01 mg/kg | < 0.01 mg/kg |
| Malathion | < 0.01 mg/kg | < 0.01 mg/kg |
| Parathion | < 0.01 mg/kg | < 0.01 mg/kg |

## Figures

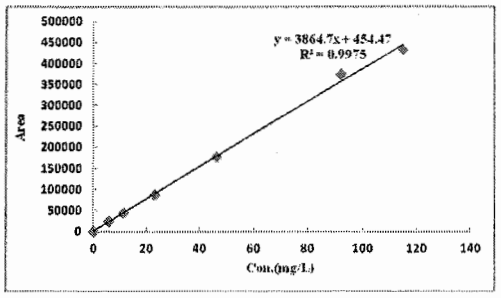


**Figure 1 Calibration Curve for the test substance**

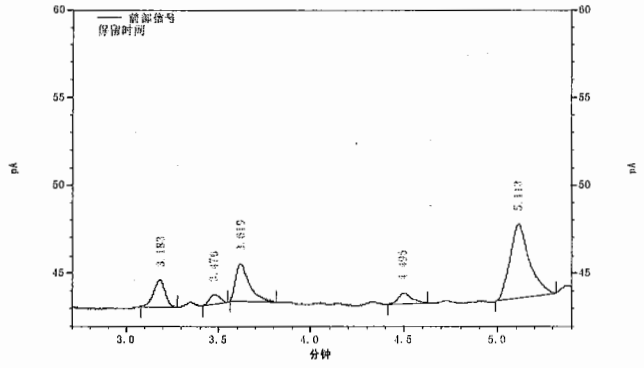


**Figure 2 Standard GC-FID Chromatogram of the test substance (115 mg/L)**

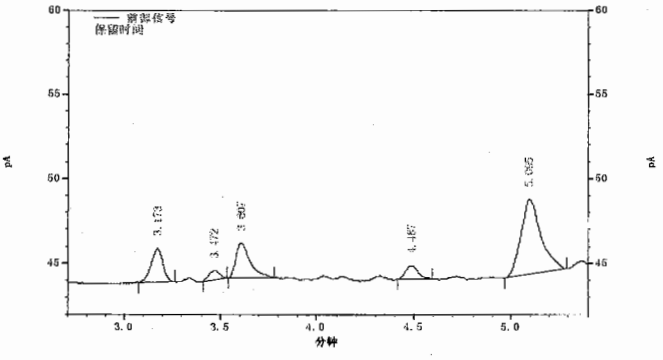


**Figure 3 GC-FID Chromatogram of the Recovery Test Sample with Concentration of 1.15 mg/L (100-fold concentrate)**

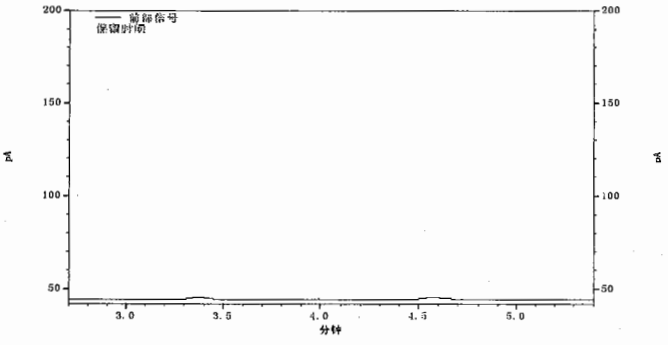


**Figure 4 GC-FID Chromatogram of the Control Sample (Definitive Test)**

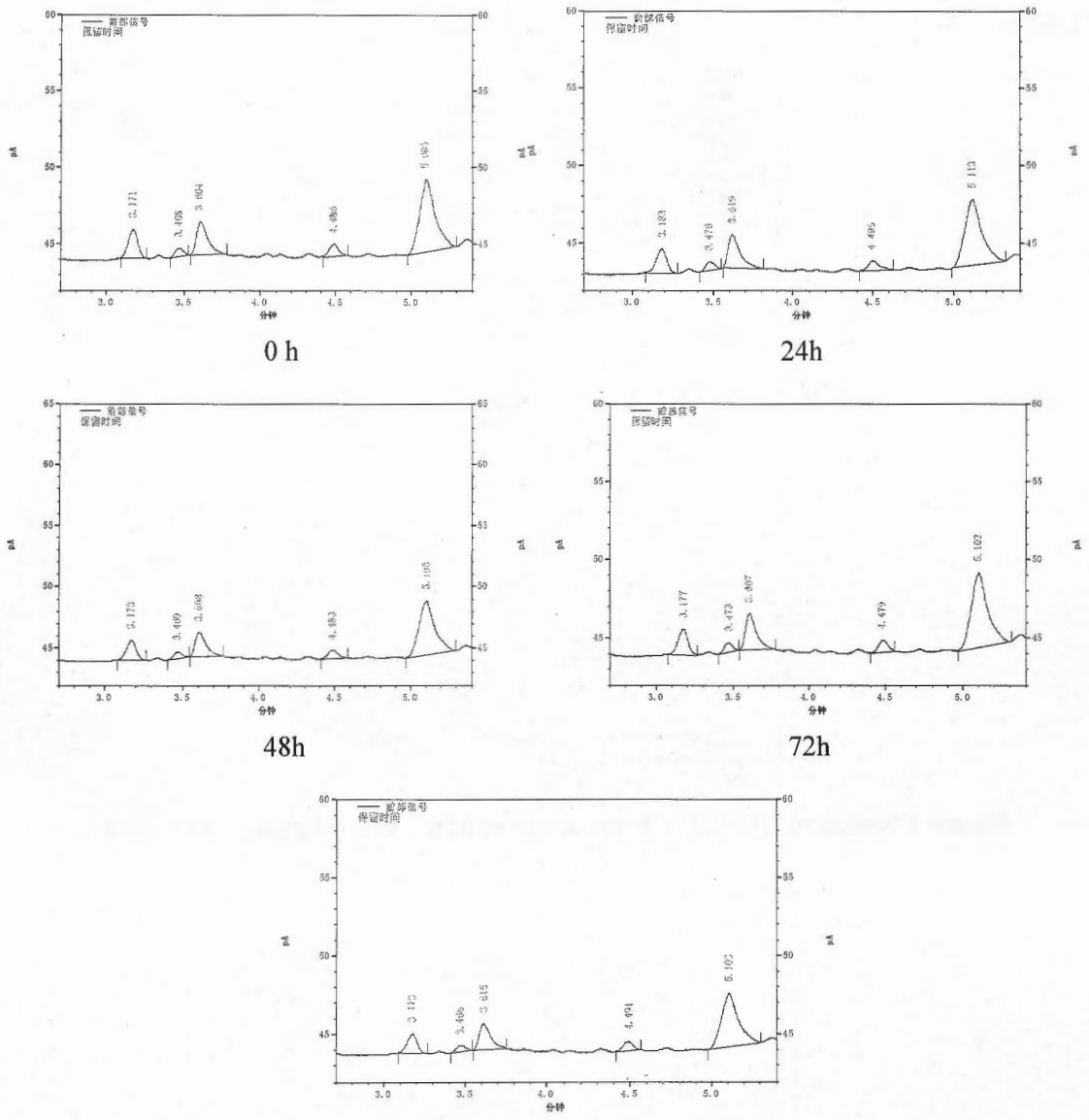


**Figure 5 GC-FID Chromatograms of the test substance with Concentration of 10% stock solution (100-fold concentrate)**

# Annex I Certificate of Analysis of the Test Substance

**Chemtura Corporation**
12 Spencer St
Naugatuck, CT 06770

Analytical Services
www.chemtura.com

## Certificate of Purity

Customer: Support for Toxicology Studies

Test Substance Name: MLA3202; Amides, tallow, N,N-bis(2-hydroxypropyl)

Physical Appearance: Liquid

CAS No.: 1454803-04-3

Ref. or Lot Number: RC-1045

Date of Analysis: revised March 18, 2016 (original issue March 7, 2016)

| Percent Composition | Monoisotopic Mass (daltons) | Formula | Structure/ Identity |
|---|---|---|---|
| 33.1 | 397.4 | $C_{24}H_{47}NO_3$ | C18:1 (oleic) tallow amides, N,N-bis(2-hydroxypropyl) |
| 22.9 | 371.3 | $C_{22}H_{45}NO_3$ | C16:0 (palmitic) tallow amides, N,N-bis(2-hydroxypropyl) |
| 13.6 | 395.4 | $C_{24}H_{45}NO_3$ | C18:2 (linoleic) tallow amides, N,N-bis(2-hydroxypropyl) |
| 11.0 | 399.4 | $C_{24}H_{49}NO_3$ | C18:0 (stearic) tallow amides, N,N-bis(2-hydroxypropyl) |
| 6.0 | 369.3 | $C_{22}H_{43}NO_3$ | C16:1 (palmitoleic) tallow amides, N,N-bis(2-hydroxypropyl) |
| 3.2 | 419.3 | $C_{26}H_{45}NO_3$ | C20:4 (eicosatetraenoic) tallow amides, N,N-bis (2-hydroxypropyl) |
| 2.0 | 393.3 | $C_{24}H_{43}NO_3$ | C18:3 (linolenic) tallow amides, N,N-bis(2-hydroxypropyl) |
| 1.5 | 282.3 | $C_{18}H_{34}O_2$ | C18:1 (oleic) acid |
| 1.1 | 421.4 | $C_{26}H_{47}NO_3$ | C20:3 (eicosatrienoic) tallow amides, N,N-bis (2-hydroxypropyl) |
| 5.6 | | | Sum of residual components (< 1% each) |
| 100.0 | | | Total |

Blake Lewis 3/7/16
Blake Lewis Date
Analytical REACh Scientist, Analytical Services

Colin Moore for ABN 3/7/16
Albert J. Nitowski Date
Sr. Technology Manager
Analytical and Lab Support Services